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Anti-UV Activity of *Lentinus edodes* Mycelia Extract (LEM)

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Abstract. *Background: Using our recently established simple method for evaluating protective activity from ultraviolet ray injury (referred to as 'anti-UV activity'), the effectiveness of various antioxidants and plant extracts was investigated. Materials and Methods: HSC-2 human oral squamous cell carcinoma cells were exposed to UV irradiation (wavelength: 253.7 nm, 6 J/m^2) in phosphate-buffered saline (PBS(–)) containing various concentrations of samples and then incubated for 48 hours in regular culture medium to determine the viable cell number by the MTT method. Results: Among the representative antioxidants, sodium ascorbate showed the most potent anti-UV activity, whereas catalase and N-acetyl-L-cysteine were inactive. Lentinus edodes mycelia extract (LEM) showed comparable anti-UV activity to sodium ascorbate. Hot water extracts of green tea and coffee, and PET-bottled of green tea extract showed slightly less, but noticeable anti-UV activity. On the other hand, hot water extracts of black tea and Jasmine tea, and PET-bottled of oolong tea, barley tea and Kohki tea were inactive. LEM was separated by gel filtration chromatography into four fractions from high to low molecular weight: polysaccharide, large and small lignin–carbohydrate complexes, and sugars. Anti-UV activity was shown by the lignin–carbohydrate fractions, but not the polysaccharide and sugar fractions. LEM, at high concentration, slightly enhanced the anti-UV activity of sodium ascorbate. Conclusion: LEM may be applicable as a UV-protective agent.*

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Ultraviolet rays (UV) are invisible electromagnetic waves with wavelength ranging from 1-400 nm, and classified into UVA (400-315 nm), UVB (315-280nm) and UVC (<280 nm). UVA and UVB pass through the ozonosphere and reach the ground, whereas UVC cannot pass through the air due to absorption. Ninety nine percent of the UV that reaches the ground is UVA. Moderate doses of UV exert several favorable effects such as sterilization and disinfection (1), vitamin D synthesis (2), stimulation of the metabolism and skin resistance. However, excessive doses of UV produce reactive oxygen species (ROS), which damage cellular DNA and proteins, leading to carcinogenesis (3). Guanine, the most susceptible DNA base, is oxidized to 7,8-dihydroxy-8-oxoguanine upon UV irradiation, and triggers the transversion of G:C to T:A bases (4, 5).

High dose of UV irradiation induced apoptotic cell death in human myelogenous leukaemia cell lines, but induced other types of cell death in human T-cell leukaemia, erythro-leukaemia, glioblastoma (6), oral squamous cell carcinoma (OSCC) cell lines and human normal oral cells (gingival fibroblasts, pulp cells, periodontal ligament fibroblasts) (7).

We recently established a method that can measure the activity of a substance in protecting cells from UV-induced injury (referred to as 'anti-UV activity') (7, 8). Using this method, the anti-UV activity of various antioxidants, beverage and plant extracts was investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (–)epigallocatechin gallate (EGCG), catalase, *N*-acetyl-L-cysteine (NAC) (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chemical Ind., Osaka, Japan); sodium ascorbate, gallic acid (Tokyo Chemical Industry Co., Ltd., Tokyo). PET bottles of green tea, oolong tea (Suntry Holding Ltd., Tokyo, Japan), and barley tea (Itoen Inc.,

Shizuoka, Japan) were purchased in a convenience store. Kohki tea was provided by Maruzen Pharmaceuticals Co., Ltd., Hiroshima, Japan.

Cell culture. Human OSCC HSC-2 cells (provided by Professor M. Nagumo, Showa University) were used since this cell line is very sensitive to UV irradiation as compared with human oral normal cells (gingival fibroblast, pulp cells, periodontal ligament fibroblasts) (7, 8). The cells were cultured at 37° in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate under humidified 5% CO_2 atmosphere.

Preparation of Lentinus edodes (LEM), beverage and plant extract. *Lentinus edodes* mycelia (HumaLab, Tokyo, Japan) were sterilely inoculated on to solid media comprised of bagasse (sugar cane fiber) and defatted rice bran and cultured for 7 months under constant temperature, humidity and light intensity. Before sprouting, the entire culture medium was ground, digested with enzymes, extracted with hot water and then purified, as described previously (9). *Lentinus edodes* mycelia extract (LEM) is a brownish powder, certified as a non-toxic material, based on the toxicity test after repeated oral administration and chromosomal aberration test. LEM contains phenylpropenoids (*p*-coumaric acid, ferulic acid, caffeic acid), vanillic acid, syringic acid, vanillin, *p*-hydroxybenzoic acid, amino acid, lignin–carbohydrate complex, β-glucan, eritadenine, and vitamins as functional components (10).

Green tea (Sayamacha, Saitama, Japan), black tea (Lipton), Jasmine tea and coffee (Nescafe) were extracted for 30 min with 3 volumes of water at 90˚C, and centrifuged for 5 min at 15,000 ×*g* at 25˚C to collect the supernatant (extract).

UV protection assay. The cells were inoculated at 3×10^3 cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Becton Dickinson Labware, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 ml of phosphate-buffered saline [PBS(–)] to minimize the evaporation of water from the culture medium. After 48 hours, the media of the cells attached to the microwell plates were replaced with PBS(–) containing sample. PBS(–), instead of DMEM+10%FBS, was used for the medium during UV irradiation, since DMEM+10%FBS contains radical scavengers or UV absorbing substances such as phenol red and proteins (7, 8). The near confluent cells were then placed at 20.5 cm distance from a UV lamp (wavelength: 253.7 nm) and exposed to UV irradiation (6 J/m^2/min) for 1 min (Figure 1), unless otherwise stated. The media were replaced with fresh DMEM+10% FBS and the cells were cultured for a further 48 hours at 37˚C in a CO_2 incubator before the relative viable cell number was determined by the MTT method. In brief, the treated cells were incubated for another 4 hours in fresh culture medium containing 0.2 mg/ml MTT. The cells were then lysed with 0.1 ml of DMSO and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the concentration that increased the viability of the UV-irradiated cells to 50% of the control value (EC_{50}) were determined. The selectivity index (SI) was determined by the following equation: $SI=CC_{50}/EC_{50}$ (7, 8).

Fractionation of LEM extract by gel filtration chromatography. LEM powder (5 g) was suspended in 20 mM $NaHCO_3$ (50 ml) and dispersed under ultrasonication for 5 min. This extract (600 μl) was applied to a TOYOPEARL HW-55F column (1.5 cm *i.d.* × 100 cm; TOSOH, Tokyo, Japan), and the column was eluted with 20 mM $NaHCO_3$ at 0.3 ml/min. The eluate was collected every 10 min and the elution curve made by plotting the eluate absorbance at 254 nm. The sugar and polyphenol contents in each fraction were measured by the phenol-sulfuric acid method and Folin-Denis method. In brief, for the phenol-sulfuric acid method, 25 μl of the eluate, 25 μl of 5% phenol (v/w) and 125 μl of sulfuric acid were mixed. The mixture was left to stand for 30 min at room temperature, and the absorbance at 490 nm of the mixture was determined using a microplate reader. For the Folin-Denis method, 25 μl of the eluate, 25 μl of 2 N phenol reagent and 125 μl of 1 N NaOH were mixed. The mixture was left to stand for 30 min at room temperature, and the absorbance at 630 nm of the mixture was determined using a microplate reader.

Results

Optimal condition of UV irradiation. Sodium ascorbate dose-dependently inhibited the UV-induced cytotoxicity in a bell-shaped fashion (Figure 2). The potency of anti-UV activity of sodium ascorbate depended both on the irradiation time and the volume of PBS(–). When the irradiation time was 1 min and the volume of PBS(–) was 100 μl, all the cells died after 48 h incubation in regular culture medium (DMEM+10% FBS) (Figure 2A). The cell viability was restored to 50% by the addition of 0.28 mM (EC_{50}) sodium ascorbate during the UV irradiation. Higher cell viability was maintained until the concentration of ascorbate reached 29.8 mM (CC_{50}), yielding the SI (CC_{50}/EC_{50}) value of 106 (Figure 2A). A slight increase of anti-UV activity (SI=148) was observed when the volume of PBS(–) was increased from 100 μl to 200 μl (Figure 2B). When the irradiation time was extended to 2 min, the protective effect of sodium ascorbate was observed only in 200 μl of PBS(–) but not in 100 μl PBS(–) (Figure 2D and C). Based on these results, the irradiation time and the volume of PBS(–) were set to 1 min and 100 μl, respectively, in the following experiments.

Anti-UV activity of antioxidants and LEM. Among the antioxidants (Figure 3), sodium ascorbate showed the most potent anti-UV activity (SI=42.4), followed by gallic acid, a component of tannin (SI=17.1) and EGCG, a major component of green tea (SI=7.7) (Figure 3A-C). On the other hand, popular antioxidants, NAC and catalase were unable to prevent the UV-induced cellular damage (cell viability <12.7%; Figure 3D and E). LEM showed anti-UV activity (SI=41.9) comparable with that of sodium ascorbate (F).

Anti-UV activity of tea, coffee and plant extracts. Hot water extracts of green tea (SI=3.4) and coffee (SI=9.6) showed some anti-UV activity, whereas those of black tea (SI<0.03) and jasmine tea (SI<0.1) were inactive (Figure 4).

Commercially available PET-bottled green tea showed some anti-UV activity (SI=1.6), whereas PET-bottled oolong tea (SI<0.9), barley tea (SI<1) and Kohki tea (SI<0.9) were all inactive (Figure 5).

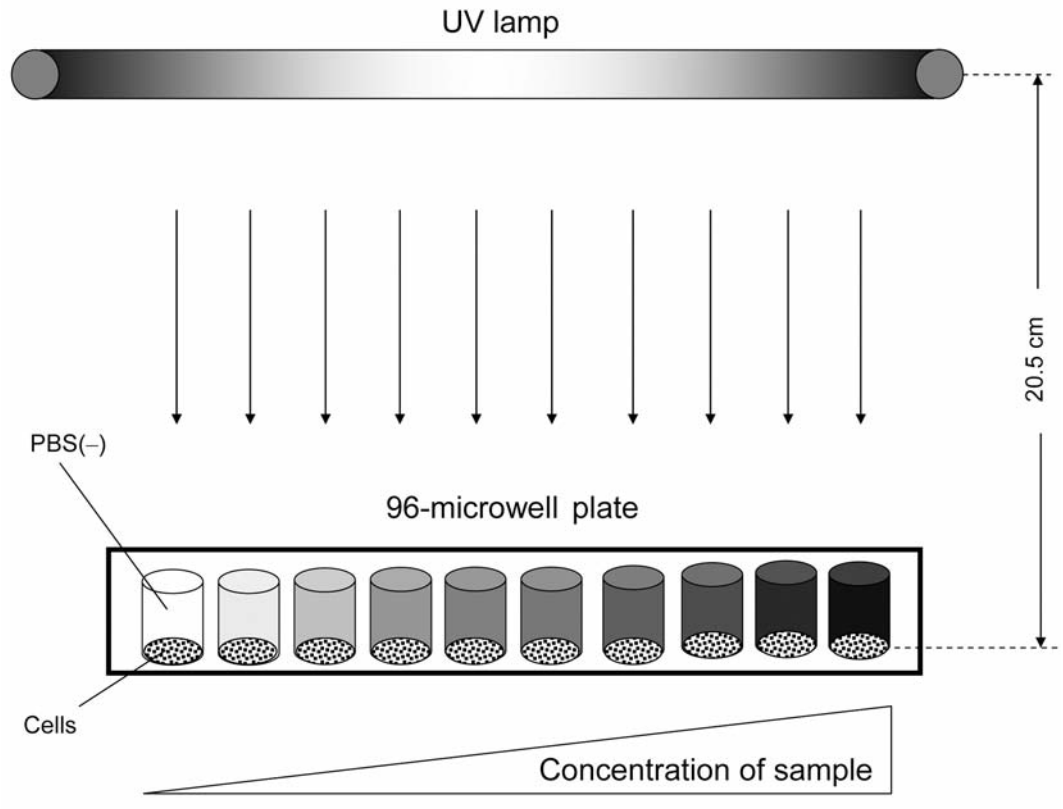

Figure 1. *Experimental protocol of UV irradiation.*

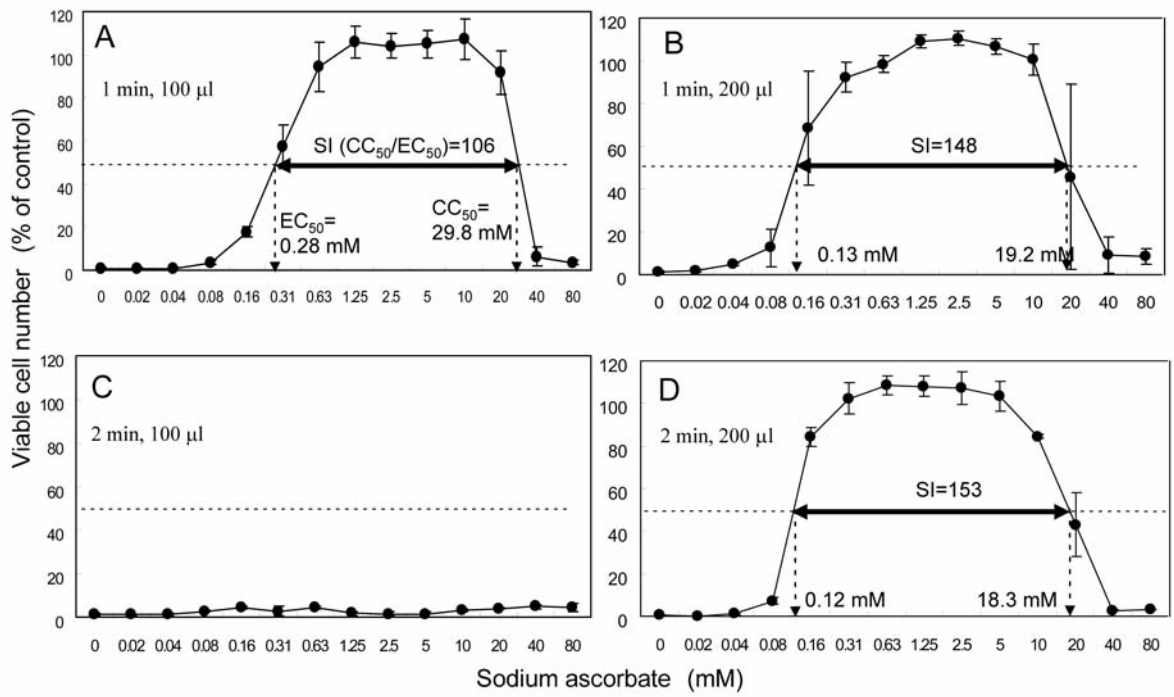

Figure 2. *Optimal UV irradiation time and PBS(–) volume. HSC-2 cells were exposed to UV irradiation for 1 (A, B) or 2 (C, D) minutes in 100 (A, C) or 200 (B D) µl of PBS (–) that contained the indicated concentrations of sodium ascorbate. Viable cell number was determined by MTT method after subsequent 48 hours culture in medium. Mean±S.D. (n=3).*

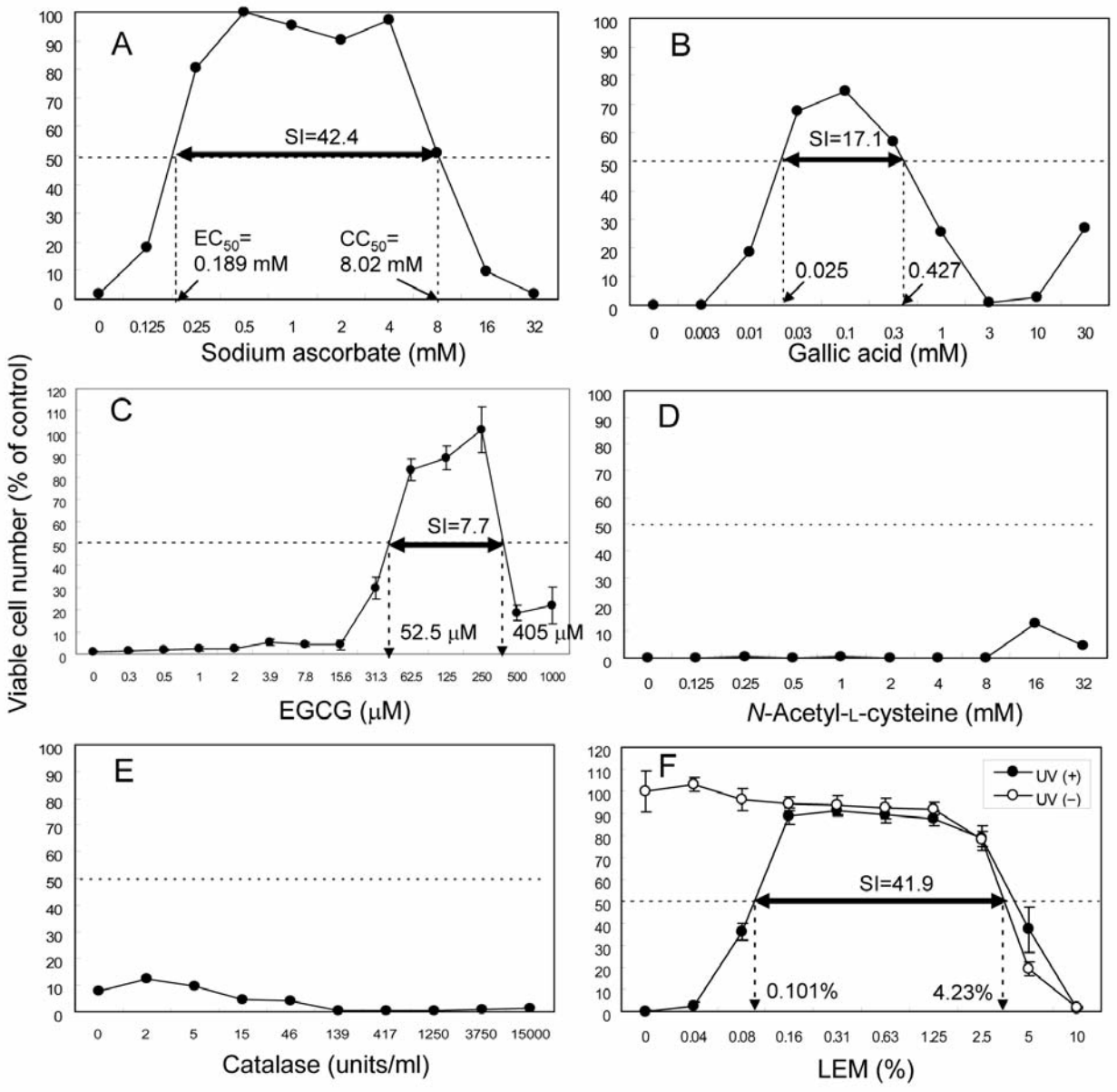

Figure 3. *Anti-UV activity of antioxidants and LEM. HSC-2 cells were exposed to UV irradiation (●) or not (○) for 1 minute in PBS(–) containing the indicated substances. Viable cell number was determined by MTT method after subsequent 48 hours culture in medium. Mean±S.D. (n=3).*

Combination effect of LEM and sodium ascorbate. High concentration (500 μg/ml), but not low concentration (250 μg/ml) of LEM enhanced the anti-UV activity of sodium ascorbate (Figure 6).

Anti-UV activity of LEM fraction. LEM was separated by gel filtration chromatography into four fractions: polysaccharide (fractions 25-29 [75-87 ml]), lignin–carbohydrate complex (fractions 30-43 [90-129 ml] and 44-48 [132-144 ml]) and low molecular sugars (fractions 49-57 [147-171 ml]) (Figure 7A). Under the standard UV irradiation conditions, all the HSC-2 cells died. When each LEM fraction was added during UV irradiation, the viability of the cells was restored to various extents, depending on the elution fraction (Figure 7B). Most of the anti-UV activity was shown in the two peaks of the lignin–carbohydrate complex fractions. None of the gel filtration fractions significantly affected the growth of the HSC-2 cells which were not exposed to UV irradiation (data not shown).

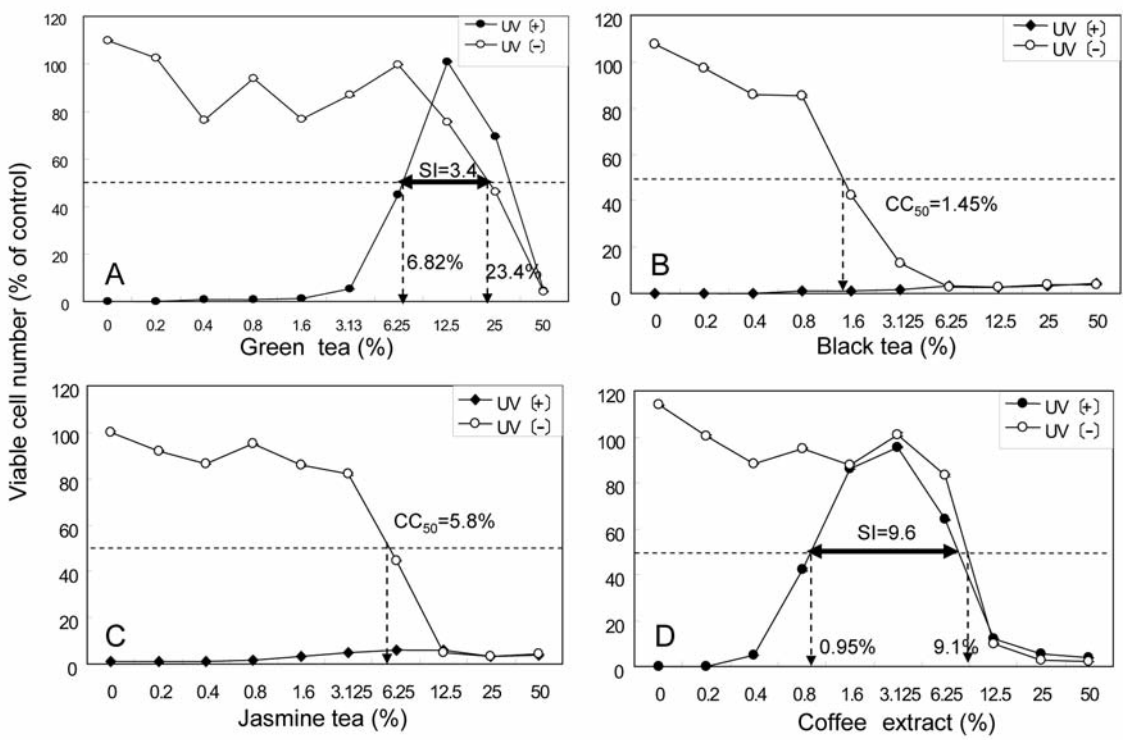

Figure 4. *Anti-UV activity of beverage extracts. HSC-2 cells were exposed to UV irradiation (●) or not (○) for 1 min in PBS(–) containing the indicated beverage concentrations, isotonized with 9 mg/ml NaCl. Viable cell number was determined by MTT method after 48 hours' culture in medium. Mean±S.D. (n=3).*

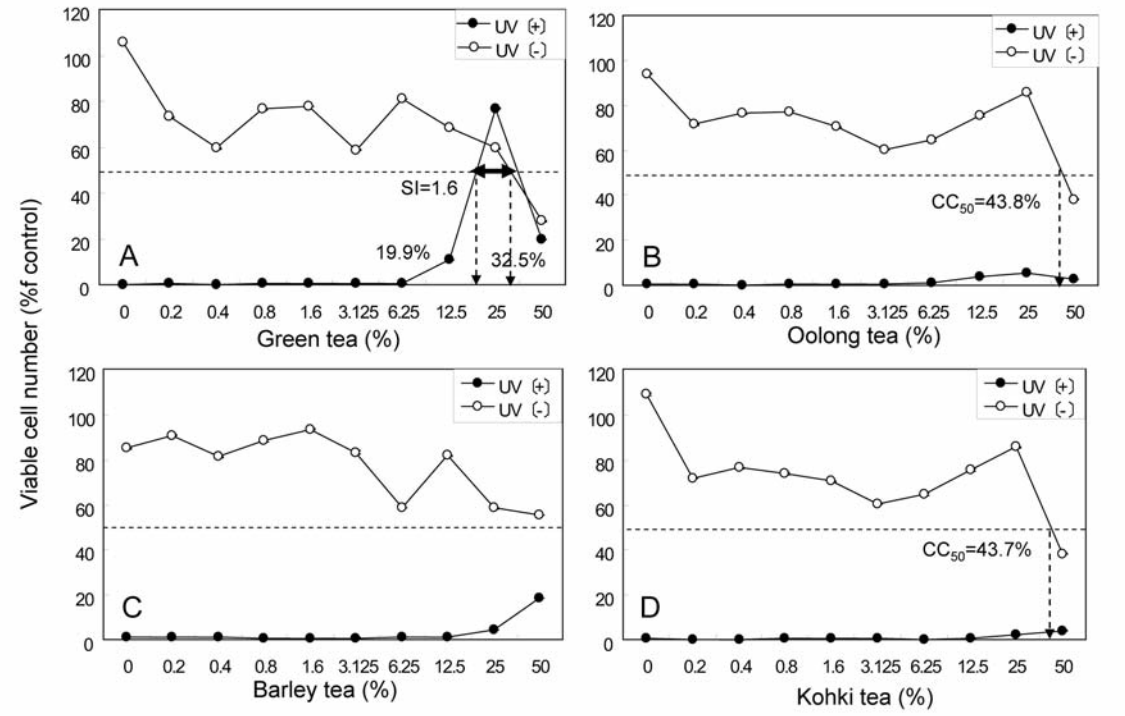

Figure 5. *Anti-UV activity of commercially available PET-bottled of beverage extracts. HSC-2 cells were exposed to UV irradiation (●) or not (○) for 1 min in PBS(–) containing the indicated beverage (PET-bottled) concentrations, isotonized with 9 mg/ml NaCl. Viable cell number was determined by MTT method after 48 hours' culture in medium. Mean±S.D. (n=3).*

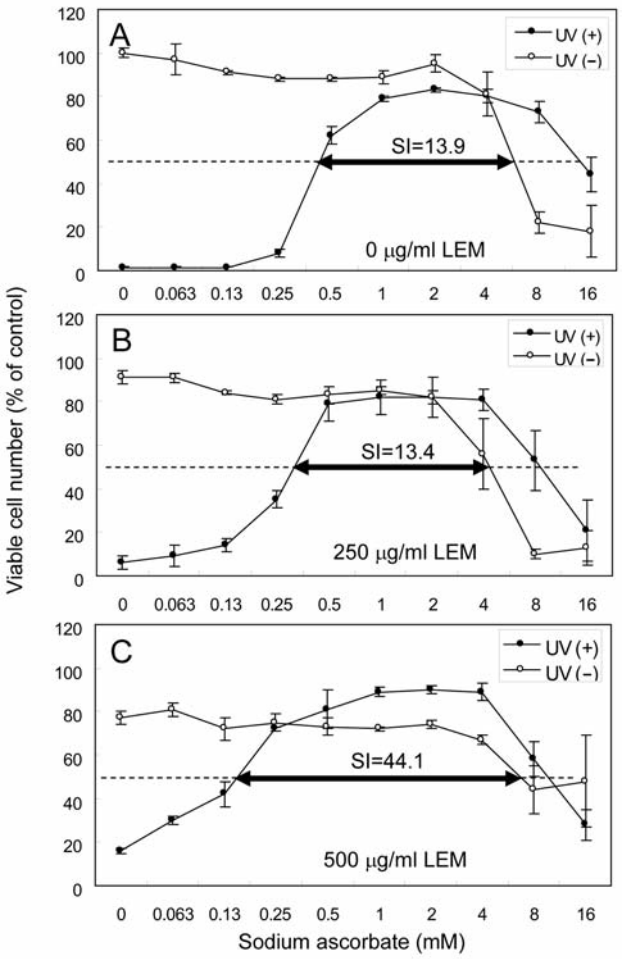

Figure 6. *Syngergistic UV-activity of LEM and sodium ascorbate. The cells were exposed or not to UV irradiation in PBS(–) without (control) or with the indicated concentrations of sodium ascorbate in the absence (A) or presence of 250 (B) or 500 (C) μg/ml LEM. The viable cell number was then determined by MTT method after a further 4 hours' incubation in medium and expressed as percent of control (without UV irradiation or LEM). Mean±S.D. of triplicate determinations.*

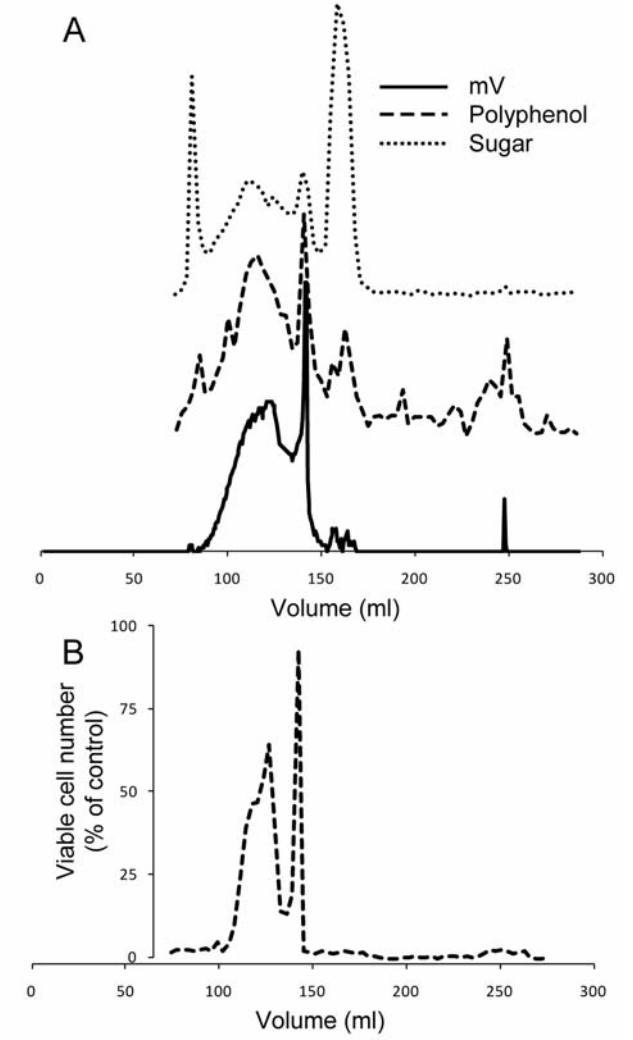

Figure 7. *Fractionated LEM. A: Fractionation of LEM (60 mg/600 μl) by gel filtration chromatography. Fraction size: 3 ml (10 min); Detection: 254 nm, mV, voltage converted from absorbance. B: Anti-UV protective activity of each fraction. HSC-2 cells were exposed to UV irradiation in PBS(–) containing 70% of each fraction. Viable cell number was determined by MTT method after 48 hours' culture in medium, expressed as percent of that of the control (without UV irradiation or LEM).*

Discussion

The present simple assay method for anti-UV activity has the following advantages. Even if the concentration of sample is unknown, the anti-UV activity can be determined from the SI value. Sterilization by autoclave and filtration through Millipore filter is not necessary for the cell culture, since the UV irradiation step that is included in the assay procedure is bactericidal. The fluctuation of SI values from experiment to experiment can be corrected by including sodium ascorbate as positive control in each experiment.

The present study demonstrated that NAC and catalase had no anti-UV activity, suggesting that H_2O_2 may not be involved in the UV-induced cytotoxicity, but the type of radical species produced by UV irradiation remains to be identified.

LEM showed comparable anti-UV activity with sodium ascorbate. At present, the mechanism of anti-UV action of LEM is unclear, but may be the result of by its radical-scavenging activity, since LEM at 150 μg/ml can scavenge 65.1% of superoxide anion radical (generated by hypoxanthine and xanthine oxidase reation) and 1500 μg/ml can scavenge 69.4% of hydroxyl radical (generated by Fenton reaction)

We have previously reported that polyphenols in LEM are mostly derived from lignin–carbohydrate complex, but not from flavonoids, tannins and related compounds (10). The lignin–carbohydrate complex from LEM also enhanced the mRNA expression of dectin-2 (4.2-fold) and toll-like receptor (TLR)-2 (2.5-fold) prominently, but only slightly modified the mRNA expression of dectin-1 (0.8-fold), complement receptor 3 (0.9-fold), TLRs-1, -3, -4, -9, -13 (0.8- to 1.7-fold), spleen tyrosine kinase, zeta-chain (TCR) associated protein kinase 70 kDa, Janus tyrosine kinase 2 (1.0- to 1.2-fold), nuclear factor (Nf)κb1, NFκb2, reticuloendotheliosis viral oncogene homolog a and b (1.0- to 1.6-fold), Nfκbia, Nfκbib, Nfκbie, Nfκbi12 Nfκbiz (0.8- to 2.3-fold) (11, 12). This suggests the possibility that the anti-UV activity of LEM may be generated by signal transduction through the dectin-2 receptor. Another possibility is that LEM may stimulate or enhance the expression of transcription factor NF-E2-related factor 2 that directs the synthesis of antioxidative enzymes such as glutathione *S*-transferase and glucose-6-phosphate dehydrogenase (13). Further study is required to test these possibilities.

The present study also demonstrated the synergistic anti-UV activity of LEM and sodium ascorbate (Figure 6), and that the majority of LEM anti-UV activity is derived from the lignin–carbohydrate fractions. This was consistent with our previous finding that LEM has several lignin-like activities (9) and that lignin–carbohydrate complex from various plant materials enhanced both the radical intensity and cytotoxicity of sodium ascorbate (10). The mechanism of synergistic action between LEM and sodium ascorbate remains to be investigated.

LEM has been reported to exert diverse biological activities such as anti-hepatopathic (14-16), antitumour (17-21), immunopotentiating (22, 23), anti-vascularisation (24) and skin-protective activities (25). Lentinan (26) and KS-2 (27), polysaccharides isolated from *L. edodes,* have immunopotentiating and antitumour activities. Lignin fractions of LEM have anti-HIV (9, 28) and immuno-potentiating (29) activity. The present study adds a new biological activity of LEM, suggesting it to be a promising candidate for alternative medicine.

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